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A possible role for cysteine residues in the fidelity of DNA synthesis exhibited by the reverse transcriptases of human immunodeficiency viruses type 1 and type 2

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HIV reverse transcriptases (RTs) have few cysteine residues relative to other RTs and retain their DNA polymerization functions following chemical modification by thiol-specific reagents. The functional role of the cysteines in the fidelity of the DNA-dependent DNA synthesis of HIV RTs has been addressed by chemical modification of the wild-type enzymes in combination with the analysis of an enzymatically active mutant HIV-1 RT in which all cysteines were modified to serines. We have observed an increase in 3'-terminal mispair extension efficiency exhibited by chemically modified HIV-1 and HIV-2 RTs. The possible involvement of cysteine residues was further substantiated using the cysteine-free mutant HIV-1 RT that displays an increased efficiency of mispair extension. These results provide evidence for a possible role of cysteine residues in the fidelity of DNA synthesis catalyzed by HIV RTs.

Fidelity; DNA synthesis; HIV; RT; Cysteine

1. INTRODUCTION

Human immunodeficiency viruses type 1 and 2 represent a distinct group of lentiviruses associated with acquired immunodeficiency syndrome (AIDS) [1,2]. An important characteristic of HIV-1 and HIV-2 is their high genomic diversity [3–5]. It has been suggested that much of this genetic heterogeneity stems from the low fidelity of the reverse transcription step [6]. HIV-1 RT was shown to be significantly more error-prone than other retroviral RTs due to its ability to elongate efficiently mismatched 3' termini of the primer DNA [7]. Our recent studies on fidelity of HIV-2 RT revealed that this enzyme extends 3' mispaired termini as efficiently as HIV-2 RT (Bakhanashvili and Hizi, submitted).

Studies on the catalytic properties of HIV-1 and HIV-2 RTs revealed that the DNA polymerizing functions of both enzymes are completely resistant to sulfhydryl reagents [8], despite the fact that RTs are known to be sulfhydryl requiring enzymes [6]. This peculiar feature may result from the exceptionally low cysteine content of these RTs relative to other retroviral RTs. HIV-1 RT has only 2 cysteines (at positions 38 and 280 from the amino terminus) and HIV-2 RT has 3 cysteines (at positions 38, 280 and 445) out of a total of 560 and 559 amino acid residues in HIV-1 and HIV-2 RTs, respectively. In contrast, MLV RT (with 671 amino acids) has 8 cysteines while avian sarcoma virus and avian myeloblastosis/sarcoma virus RTs have 12 cysteines in the β subunit (895 residues long) and 8 cysteines in the α subunit (572 residues long) [9]. Interestingly, 'cysteine rich' RTs, that are sensitive to sulfhydryl reagents, were shown to have a relatively higher fidelity of DNA synthesis than HIV-1 RT [10]. Cysteine residues were found to be involved in the biological functions of various enzymes [11,12]. Therefore, we examined the possibility that the low fidelity of HIV RTs may be associated with their low cysteine content. The functional role of cysteine residues in both HIV RTs has been addressed by chemical modification of native enzymes in combination with analysis of an enzymatically active mutant HIV-1 RT that lacks cysteine residues. The results presented herein demonstrate that cysteine residues of HIV-1 and HIV-2 RTs might be involved in the fidelity of the DNA-dependent DNA synthesis.

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2. MATERIALS AND METHODS

2.1. *Enzymes*

All RTs wild type and mutant used in this study were recombinant enzymes expressed by us in *E. coli* [13–15] and purified to homogeneity according to Clark et al. [16]. The specific activities of the different RTs used were 4,000–5,000 units per μg. One unit is defined as the amount of enzyme that catalyzes the incorporation of 1 pmol dTMP into DNA in the poly(rA)$_n$ · oligo(dT)$_{12-18}$-directed reaction in 30 min at 37°C (20).

2.2. *Template primers*

The template single-stranded ϕx174am^3 DNA was primed with a

twofold molar excess of the 16mer oligonucleotide, that hybridizes to the nucleotides at positions 587 to 602 of the ϕx174am^3 DNA. Four versions of the 16-base primers were synthesized separately. They are identical except that the 3' terminal base (N) contains either an A, C, G or T (5' AAAGCGAGGGTATCCN 3') (see Chart 1). The primers were 5'-end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and annealed to the template DNA as described [17].

2.3. *DNA polymerization reaction*

The DNA polymerization reactions for HIV RTs contained 20 mM Tris-HCl (pH 7.5), 2 mM DTT, 10 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, primed DNA and various concentrations of dATP. For MLV RT, the reactions were carried out in the presence of 0.5 mM $MnCl_2$ substituting for $MgCl_2$. Reactions were incubated at 30°C for 10 min. Aliquots (5 μl) were removed into 5 μl of formamide dye mix, denatured at 100°C for 5 min, cooled on ice and analyzed by electrophoresis through 20% polyacrylamide gels and quantified by densitometric scanning of gel autoradiographs. Before measuring the kinetic constants for elongating the primers, a time-course study was done for each paired and mispaired terminus to determine the range of time during which the products accumulate linearly with time.

3. RESULTS AND DISCUSSION

Since extension of mismatched 3' DNA termini was found to be a major determinant of the infidelity of both HIV RTs, we analyzed this parameter as representing the fidelity of DNA synthesis. We used the four template-primer substrates as described in Chart 1. The four 16-base oligonucleotide primers were identical except that each has a different 3'-terminal nucleotide A, C, G, or T (designated as N). Consequently, the A:A, A:C, or A:G mispairs and the A:T correctly paired 3' terminus were produced at position 587 by hybridizing the primer oligonucleotides to the ϕx174am^3 DNA template.

We examined the possibility that the low fidelity of HIV-1 and HIV-2 RTs might be associated with their low cysteine content. To test this, the number of free cysteines was reduced by either chemical modifications or by site-directed mutagenesis and the fidelity of the modified HIV RTs was assayed. MLV RT could not serve as a control, because it is totally inactivated by the thiol reagents [8]. Both HIV RTs were modified by either one of the thiol-specific reagents: *N*-ethylmaleimide (NEM) or iodoacetamide (IAA). The catalysis of the extension from preformed mispairs by the HIV RTs was studied by measuring primer elongation in a DNA-dependent DNA polymerization reaction using dATP as the only deoxynucleoside triphoshate. There is a signif-

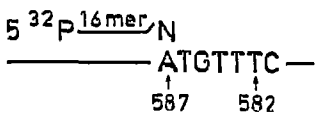

Chart 1. Primer–template used for analysis of terminal mismatch excision and for measuring extension kinetics for matched and mismatched primer 3'-terminal bases. The primer is 16 nucleotides long with 3'-terminal nucleotide N, representing A, C, G or T, and annealed to a complementary section of ϕx174am^3 DNA template.

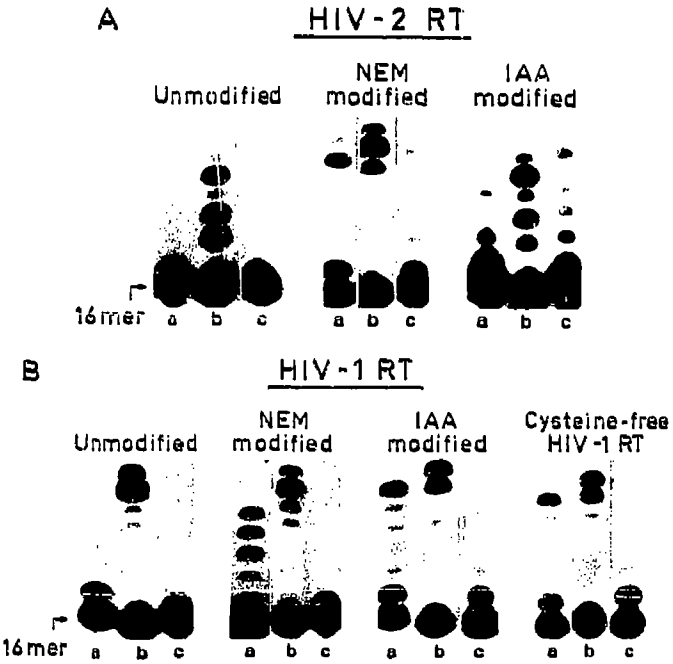

Fig. 1. Mispair extension by chemically modified HIV-1 and HIV-2 RTs and mutant cysteine free HIV-1 RT. Oligonucleotide primers were hybridized to ϕx174am^3 DNA (0.022 pmol) to produce 3'-terminal mispairs at position 587 and extended with equal activities of either HIV-2 RT (A), HIV-1 RT or cysteine-free mutant HIV-1 RT (B). In the case of mispair extension with modified enzymes, RTs were preincubated with 10 mM NEM or 10 mM IAA for 30 min on ice. The extension reactions from A:A (a), A:C (b) and A:G (c) mispairs were carried out in the presence of 1 mM dATP as described in section 2.

icant difference in mispair extension efficiency between the wild-type HIV RTs and the enzymes chemically-modified by NEM or IAA (Fig. 1). In the case of HIV-1 RT, the chemically modified enzyme extends the preformed A:A mismatch more efficiently than did the unmodified enzyme as evident by the increase in the length of the oligonucleotide primers from 16 to 22 nucleotides (Fig. 1B, lanes a). In the case of HIV-2 RT the modified enzyme exhibits an increase in extension efficiency from A:C and A:G mispairs as well as from the A:A mispair (Fig. 1A, lanes b and c). The similar pattern of mispair extension obtained by HIV-2 RT with both modifiers implicates cysteines as the major target for the chemical modification. Therefore, we further analyzed the efficiency of mispair extension by IAA-modified HIV-2 RT as representing the sulfhydryl-modified enzyme. The kinetics of mispair extension as a function of increasing concentration of dATP are shown in Fig. 2. Extension rates from all mispairs were estimated from the double reciprocal curves of V vs. [S] and the kinetic parameters are summarized in Table I. The apparent K_m values for nucleotide addition onto the A:A, A:C and A:G mispairs were 1,000- to 3,000-fold greater than those observed with the correctly paired A:T terminus. However, the apparent V_{max} values calculated for extension from the mispairs were 10-fold lower than those ob-

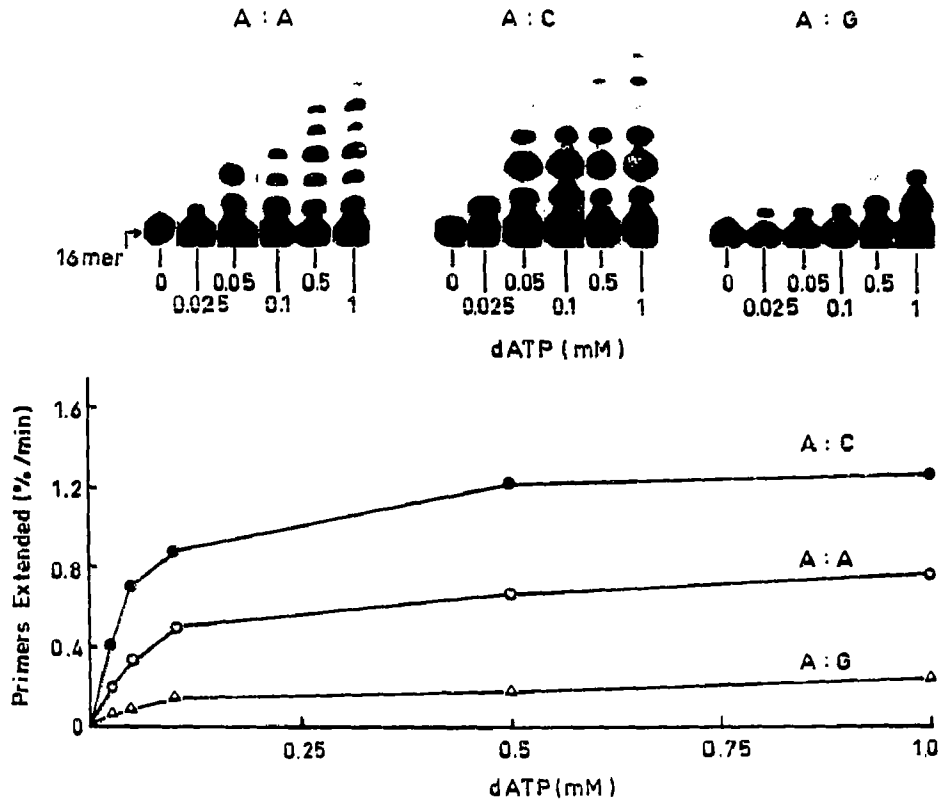

Fig. 2. Kinetics of mispair extension by iodoacetamide modified HIV-2 RT. Oligonucleotide primers were hybridized to ϕx174am^3 DNA (0.022 pmol) to produce the indicated 3'-terminal mispairs at position 587 and extended by IAA-modified HIV-2 RT. HIV-2 RT was modified by preincubation with 10 mM IAA for 30 min on ice. Each extension reaction contained 0, 0.025, 0.05, 0.1, 0.5 or 1 mM dATP as the only deoxynucleoside triphosphate substrate. Polyacrylamide gel electrophoretic analysis of the extended primers from A:A (○), A:C (●) and A:G (△) mispairs are shown in the upper panel at left, center and right, respectively. The primer position is indicated by an arrow.

served with the correctly paired terminus. As with unmodified HIV-2 RT, the efficient extension of mispairs with IAA-modified HIV-2 RT was dominated by a K_m effect. The extension frequencies were defined as the ratios of apparent V_{max}/K_m values. The ratio of mispair extension efficiency of IAA-modified HIV-2 RT relative to that of the unmodified RT indicates the greatest increase in extension frequency (by about 8-fold) from the A:A mispair. Indeed, while the extension frequency from A:A mispair with HIV-2 RT was 1/23,000, it appeared to be 1/2,800 with the modified enzyme. Furthermore, the extension frequency from the A:G mispair was about 3-fold greater with the modified enzyme (1/12,000) than with the wild-type enzyme (1/35,000).

The enhanced extension frequency is likely to result from the modification of cysteine residues. However, being highly specific, the modification of proteins with NEM or IAA has been used to study the role of a thiol group in the function of numerous enzymes, although there are few cases in which they are not completely specific to cysteines. To further support the putative involvement of cysteines in the efficiency of mispair extension, we have studied a double mutant of HIV-1 RT, in which the only two cysteines (Cys-38 and Cys-280) were modified to serines [27]. Like the chemically modified HIV RTs, this mutant enzyme displays an increase in extension efficiency (Fig. 1B). The kinetics of mispair extension as a function of increasing concentration of dATP are shown in Fig. 3. The kinetic parameters were calculated as described above, and the results are summarized in Table II. As with the wild-type enzyme, the extension of mispairs by the mutant enzyme was manifested through the differences in apparent K_m values, calculated for the correct and incorrect nucleotides. Furthermore, the extension frequency from A:A mispair is 3-fold greater in the presence of mutant enzyme (1/6,500) compared to wild-type enzyme (1/20,000). The extension frequency from A:G mispair is also about 2-fold greater with the mutant enzyme (1/15,000) compared to the wild-type enzyme (1/27,000). The quantitative effect of the cysteine modification on the extension of mismatches varies greatly depending on the mismatch examined (Tables I and II).

The reduced fidelity of the mutant HIV-1 RT possibly implicates cysteine residues in the accuracy of the DNA dependent DNA polymerization. Likewise, it was recently reported that tyrosine residues are involved in the fidelity of DNA synthesis of the Klenow fragment of *E. coli* DNA Pol I [19], in which the replacement of Tyr-766 by Ser led to an increased incidence of misincorpo-

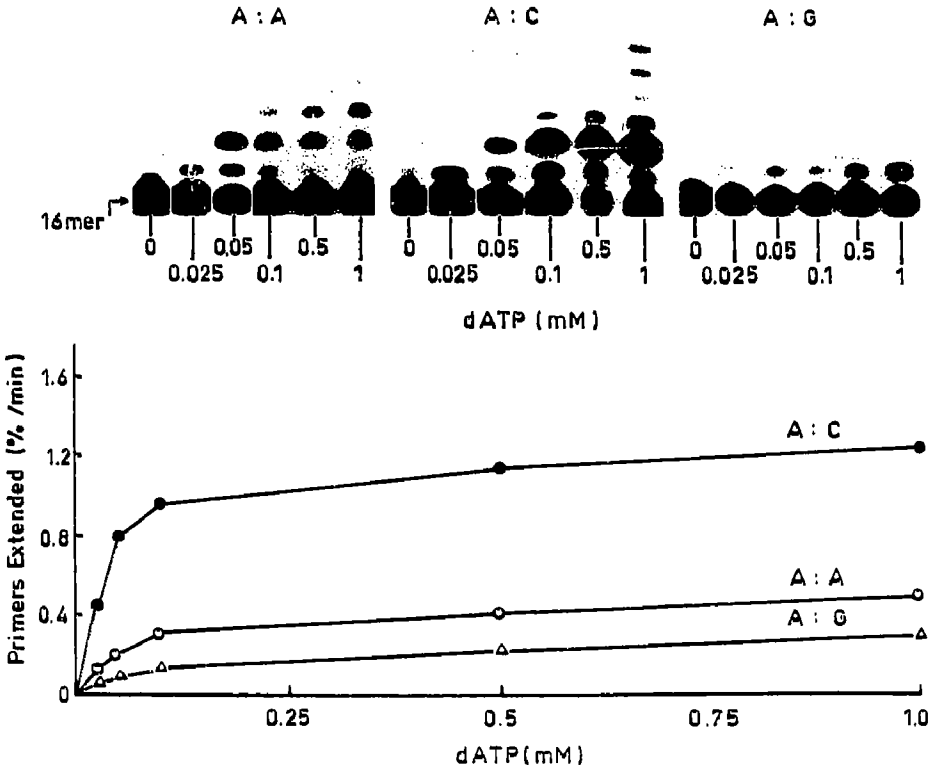

Fig. 3. Kinetics of mispair extension by cysteine-free HIV-1 RT. The experiments were conducted with cysteine-free HIV-1 RT and analyzed as described in detail in Fig. 2.

Table I

Kinetics of mispair extension by iodoacetamide-modified HIV-2 RT

Primer–template	K_m (μM)	V_{max} %/min	Relative extension efficiency	Ratio of extension efficiencies (modified/unmodified RT*)
____/A ____A–	64±7.1	0.8±0.09	1/2,800	8
____/C ____A–	40±3.8	1.3±0.07	1/1,100	2
____/G ____A–	90±19.2	0.25±0.009	1/12,000	3
____T ____A–	0.037±0.0041	1.33±0.057	1	1

ϕx174am^3 DNA was primed with 16mer oligonucleotide primers to produce the indicated 3′ termini. Extension reactions (10 min, 30°C) contained 0, 0.025, 0.05, 0.1, 0.5, or 1 mM of dATP and IAA-modified HIV-2 RT (see Fig. 2). The percentage of 16mer extended by at least one nucleotide was quantitated as described in section 2. The apparent K_m and V_{max} values ± S.E. were determined by the method of Wilkinson [18]. Relative extension frequencies are ratios of the rate constant (V_{max}/K_m) for the mispair divided by the corresponding rate constant for the paired A:T terminus. The relative extension frequencies for wild-type HIV-2 RT were calculated previously (Bakhanashvili and Hizi, submitted) and found to be 1/23,000 for A:A mispair, 1/1,900 for A:C, and 1/35,000 for A:G.

ration. The biochemical role of the cysteine residues in the mispair extension reaction is still obscure and deserves more thorough studies. Cysteines were reported to affect selective activities of several enzymes. Thus, a selective metal binding to Cys-78 of T4 endonuclease V

Table II

Kinetics of mispair extension by cysteine-free HIV-1 RT

Primer–template	K_m (μM)	V_{max} %/min	Relative extension frequency	Ratio of extension frequencies (cys-free/wild-type HIV-1 RT)
____/A ____A–	58±7.1	0.43±0.041	1/6,500	3
____/C ____A–	30±2.9	1.25±0.09	1/1,200	2
____/G ____A–	82±7.4	0.25±0.01	1/15,000	2
____T ____A–	0.034±0.0031	1.65	1	1

The extension reactions were conducted with cysteine-free HIV-1 RT (see Fig. 3) and analyzed as described in Table I. The relative extension frequencies for wild-type HIV-1 RT were calculated previously (Bakhanashvili and Hizi, submitted) and found to be 1/20,000 for A:A mismatch, 1/2,500 for A:C, and 1/27,000 for A:G.

caused an inhibition of the DNA incision performed by the enzyme [11]. Mutation of the cysteine residue in the *S. cerevisiae* RAD6 protein abolishes its ubiquitin-conjugating activity required for DNA repair [12].

There are several interesting differences in the increase in mispair extension frequency detected for the chemically-modified HIV-2 RT and the mutated HIV-1 RT. Chemically-modified HIV-2 RT extends all mismatches more efficiently than do chemically modified or mutated HIV-1 RT. These differences may be due to the involvement of the third cysteine residue (Cys-445), that is found only in HIV-2 RT. Consequently we have begun to investigate the role of each cysteine residue in the fidelity of DNA-dependent DNA polymerization using mutants of HIV-1 and HIV-2 RTs, in which the cysteines were individually modified.

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